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(54) Title: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

(57) Abstract

This invention describes a novel agent for the targeted control of a mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders. An agent according to the invention comprises three Domains B, T and E linked together in the following manner: Domain B–Domain T–Domain E where Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome, Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell, Domain E is the Effector Domain which inhibits the ability of the Recyclable Membrane Vesicles to transport the Integral Membrane Proteins to the surface of the cell.

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NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

TECHNICAL FIELD

This invention describes a novel agent for the targeted control of mammalian cell activity, in particular the agent is used to control the interaction of particular cell types with their external environment. The agent has applications as a pharmaceutical for the treatment of a variety of disorders.

BACKGROUND

A fundamental property of living cells is their ability to respond to their external environment. The interface between a cell and its external environment is the plasma membrane. The plasma membrane consists of a phospholipid bilayer in which many kinds of protein molecules are embedded. These integral membrane proteins (IMPs) are responsible for many of the interactions of a cell with its external environment.

The interactions in which the IMPs are involved include: the transport of materials, including nutrients, into and out of the cell; the regulated permeability of the plasma membrane to ions; the recognition of, and response to, extracellular molecules; and the adhesion of one cell to another cell. A specialised function of the immune system, that is also mediated via IMPs, is the display of particular foreign peptide sequences by one group of immune cells to another group.

One of the ways in which a cell regulates its ability to respond to, and interact with, the external environment is by changing the quantity and types of IMPs present at the plasma membrane. One mechanism by which this is achieved is the reversible internalisation of IMPs via an endocytotic pathway into Recyclable Membrane Vesicles (RMVs). In these cases IMPs stored in the RMVs represent an internal store or pool of IMPs available for rapid export to the cell surface via a process of exocytotic fusion of the RMVs with the plasma membrane. Modulation of the equilibrium of this exocytotic / endocytotic

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cycle allows rapid regulation of the density of IMPs present at
the cell surface. In one example of the process for controlling
cell activity, the uptake of glucose by insulin-responsive cells
in skeletal muscle and adipose tissue is regulated. Insulin
increases the amount of a particular isoform of glucose
transporter, GLUT4, which is found in the plasma membrane of
these cells. The higher concentration of GLUT4 molecules at the
surface of the cell results in increased uptake of glucose.
Therefore, by controlling the number of glucose transporters
present in the plasma membrane the response to insulin can be
modulated.

Another example of alterations in cell surface IMP expression
in response to external signals is that of the receptor for the
complement fragments C3b and C4b, the type 1 complement receptor
CR1. Upon activation of human neutrophils the plasma membrane
expression of CR1 is transiently increased 6- to 10- fold.

In another example a number of inflammatory and immune cells
modify their expression of cell surface adhesion molecules upon
activation. Hence, activation of neutrophils or monocytes leads
to a modulation of the cell surface adhesion molecules Mac-1 and
p150,95. These adhesion molecules are important in the
targeting and movement of inflammatory cells to sites of
inflammation.

In yet another example, a variety of hormones (insulin, insulin-
like growth factor, interleukin 1 and platelet-derived growth
factor) cause a rapid increase in the cell surface expression
of the transferrin receptor in a variety of cell types. The
transferrin-receptor binds diferric transferrin from the
external environment of the cell, and is thereby involved in the
uptake of iron by cells. This transferrin/transferrin-receptor
system may also play a role in the transcellular movement of
iron into the CNS across the blood brain barrier, a process
known as transcytosis. Transcytosis is also involved in the
transfer of maternal immunity to the developing foetus.

In yet another example the diuretic hormone aldosterone is known

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to increase the cell surface expression of Na⁺ channels in the
apical membrane of urinary bladder epithelial cells. This
mechanism is involved in salt retention and occurs, for example,
in conditions of low sodium-containing diets.

In a further example of the modulation of cell membrane
expression of IMPs, it is noted that the function of the immune
system is based upon the recognition of foreign, or non-self,
antigens. Part of this recognition and immune response is
provided by cells of the immune system able to recognise and
respond to foreign peptide sequences. These peptide sequences
are presented to the immune cells by other cells of the immune
system known as antigen presenting cells. Antigen presenting
cells ingest foreign antigenic proteins, digest these to
peptides and display the foreign peptides in a cleft formed at
the cell membrane by IMPs of the major histocompatibility
complex.

Thus IMPs are central to a cell's ability to interact with its
external environment and, given the diverse and varied nature
of these interactions, it is not surprising to discover that
there are a vast array of different IMPs. The pivotal role of
IMPs in a cell's function means that they are often involved in
pathophysiologic states, and are the target for many therapeutic
interventions.

Prior art approaches to the control of IMP activity have mainly
focused on modulating the function of the IMP once expressed at
the cell surface. Thus prior art therapeutic interventions tend
to be specific for particular IMPs and for particular functions
of particular cell types. Inhibitors of specific transport IMPs
have been developed as therapeutic agents. For example,
inhibitors of the 5HT transport protein of neurones are used as
anti-depressants. Antagonists of particular receptor IMPs are
very commonly used pharmaceutical agents. Examples include
antihistamines, both those specific for the H1 and the H2
subtypes of histamine receptor, and antagonists of the ß-
adrenoceptor. Inhibitors of IMP function are also widely used
as pharmaceutical agents. Examples include inhibitors of

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transmembrane ion movements such as the diuretics furosemide and amiloride, the latter of which is an inhibitor of the bladder epithelial cell apical Na$^\cdot$ channel. Inhibitors of potassium channels are known to be under development as antiarrhythmic agents. Cell adhesion IMPs are also currently targets for the development of selective antagonists.

Another approach being pursued is to selectively modify the expression of particular IMPs at the genetic level by alteration of the level of transcription of the appropriate gene coding for that IMP and hence modulation of specific IMP protein synthesis.

In summary, IMPs are known to play a critical role in the response of a cell to its external environment. Previous approaches to the control of IMPs have generally involved the targeting of a specific IMP at the cell surface and modifying its functional capacity. The control of the density of IMPs within the plasma membrane is anticipated to have broad applications in the treatment of a variety of disorders. In view of the great diversity of IMPs and the particular nature of current therapeutic interactions it is the surprising discovery of the current invention that a single class of agents can modify the expression of IMPs in a wide variety of cell types. The same class of agent is also able to modify the expression of transport IMPs, receptor IMPs, adhesion IMPs, channel IMPs and antigen presenting IMPs. Previously, agents affecting IMPs have been classified by function, for example Ca$^{\cdot\cdot}$ antagonists, the members of each group being chemically and mechanistically very diverse. The class of agent referred to in the current invention, by contrast, is structurally homogeneous, with rationally introduced substitutes of particular domains having predictable effects on the function of the agent. A further aspect of the invention is that the agent can be selectively targeted to particular types of cell to allow selective modulation of IMP expression only in that cell type.

STATEMENT OF INVENTION
The current invention relates to an agent for controlling the

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interaction of a cell with its external environment.
Specifically, the invention provides an agent for controlling
the transport of Integral Membrane Protein (IMP) molecules from
the internal components of a cell to the cell membrane, so as
to modify the cell's interaction with its external environment.
More specifically the invention provides a novel agent which
modifies the structure of Recyclable Membrane Vesicles (RMVs)
such that their ability to transport IMPs to the surface of the
cell is inhibited.

Definitions
The following terms have the following meanings;

> Integral Membrane Protein (IMP) means any protein
> which is embedded in and spans across the lipid
> bilayer of a biological membrane

> Recyclable Membrane Vesicle (RMV) means an
> intracellular vesicle, present in the cytosol of a
> cell, bounded by a lipid bilayer membrane . RMVs are
> formed from the plasma membrane and move into the
> cell interior by a process referred to as
> endocytosis. RMVs undergo a cyclical process of
> forming from and fusing with the cell plasma
> membrane. The process of moving to and fusing with
> the plasma membrane is referred to as exocytosis.
> The function of RMVs in the cell is in the
> reversible transport of IMPs to and from the cell
> surface; in this they are distinct from the
> secretory vesicles of neurosecretory cells.

> Endosome means those intracellular vesicles which
> have formed from the plasma membrane by a process of
> endocytosis.

> Heavy chain means the larger of the two polypeptide
> chains which form Clostridial neurotoxins; it has a
> molecular mass of approximately 100 kDa and is
> commonly referred to as HC. Light chain means the

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smaller of the two polypeptide chains which form
Clostridial neurotoxins; it has a molecular mass of
approximately 50 kDa and is commonly referred to as
LC. Naturally occurring Heavy and Light chains are
covalently coupled via at least one disulphide bond.

H_2 fragment means a fragment derived from the amino
terminal end of the Heavy chain of a Clostridial
neurotoxin by proteolytic cleavage for example with
trypsin or papain.

H_2L means a fragment of a Clostridial neurotoxin
produced by proteolytic cleavage for example with
trypsin or papain in which the Light chain is still
coupled via disulphide bonds to the H_2 fragment.

In one aspect of the invention an agent is provided for the
control of the level of the IMP responsible for the transport
of a metabolite across the cell membrane, so controlling the
availability of that metabolite within the cell.

In another aspect of the invention an agent is provided for the
control of the level of the IMP responsible for the transport
of a metabolite across the cell membrane into the cell and out
of the cell, so controlling the transport of that metabolite
through the cell.

In yet another aspect of the invention an agent is provided for
the control of the level of the IMP responsible for the
selective permeability of the plasma membrane of the cell to an
ion, so modulating the concentration of that ion within the
cell.

In yet another aspect of the invention an agent is provided for
the control of the level of the IMP responsible for the
recognition of a signalling molecule, so modulating the
responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for

the control of the level of the IMP responsible for the transduction of signals across the cell membrane following binding to the membrane of a signalling molecule, so modulating the responsiveness of the cell to that signalling molecule.

In yet another aspect of the invention an agent is provided for the control of the level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens. The result of this in an organism is to affect the immune response of that organism.

The invention also provides an agent which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

As previously stated, prior art approaches to the control of IMP activity have mainly focused on modulating the function of the IMP once expressed at the cell surface. In direct contrast the present invention modulates the level of IMP which becomes expressed at the cell surface.

DETAILED DESCRIPTION OF THE INVENTION
It can be seen that the object of this invention, to provide an agent for controlling the level of IMPs at a cell surface, has many potential applications for modulating the response of a cell to its environment. This invention includes an agent which functions so as to affect the mechanisms by which IMPs are carried to the surface of a cell, as evidenced in the examples, e.g. example 1 and 2. Such an agent must accomplish three discrete functions, the first two of which are known in the art. Firstly it must bind to a cell surface structure (the Binding Site). It must then enter into the cytosol of the cell. The entry of molecules into the cell is known to occur by a process of endocytosis. However, as only certain cell surface Binding Sites are known to be involved in endocytosis, only these Binding Sites are suitable as targets. Once taken into the cell by endocytosis the agent must then leave the resulting endosome across the endosomal membrane to enter the cytosol. The ability to achieve specific cell binding and entry of agents into the

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cytosol is well known in the literature (for example: Pastan,
I; Willingham, MC; & Fitzgerald, DSP, 1986, Cell 47, 641 - 648,
Olsnes, S; Sandvig, K; Petersen, OW; & Van Dews, B, 1989,
Immunol. Today 10, 291 - 295, Strom, TB; Anderson, PL; Rubin-
Kelley, VE; Williams, DP; Kiyokawa, T; & Murphy, JR; 1991, Ann
NY Acad. Sci 636, 233 -250). The third function of the agent
is the surprising finding of this invention, namely the ability
to affect the RMV. The further surprising aspect of this agent
is that by so affecting the RMV it limits its ability to
transport the IMPs to the cell surface.

The agent of the invention therefore comprises the following
functional Domains;

> Domain B, the Binding Domain, binds the agent to a
> Binding Site on the target cell capable of
> undergoing endocytosis to produce an endosome
> containing the agent

> Domain T, the Translocation Domain, translocates the
> agent or part of the agent from within the endosome
> across the endosomal membrane into the cytosol of
> the cell

> Domain E, the Effector Domain, inhibits transport of
> IMPs to the surface of the cell by RMVs.

Domain B can be made to have specificity for a target cell type.
The ability to target an agent to a particular cell type is well
known in the art. Thus, the functions of Domain B could be
achieved by the use of one of many cell-binding molecules known
in the art including, but not limited to, antibodies, monoclonal
antibodies, antibody fragments (Fab, F(ab)'$_2$, Fv, single chain
antibodies, etc.), hormones, cytokines, growth factors and
lectins.

The functions of Domain T could be achieved by mol cules capable
of forming appropriate pores within the endosomal membrane. It
is well documented that certain parts of toxin molecules are

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capable of forming such pores including, amongst others, fragments of anthrax toxin, botulinum toxin, tetanus toxin, Diphtheria toxin and Pseudomonas endotoxin (Hoch, DH; Romero-Mira, M; Ehrlich, BE; Finkelstein, A; Das Gupta, BR; & Simpson, LL; 1985 PNAS 82 1692 - 1696, Olsnes, S; Stenmark, H; Moskaug, JO; McGill, S; Madshus, IH; & Sandvig, K, 1990, Microbial Pathogenesis 8, 163 - 168.) One such molecule is the Heavy chain of clostridial neurotoxins for example botulinum neurotoxin type A. Preferably it has been found to use only those portions of the toxin molecule capable of pore-forming within the endosomal membrane.

The functions of Domain E, the inhibition of the ability to transport the IMPs to the surface of the cell are not known to the art. Surprisingly, it has been found that different portions of certain toxin molecules - functionally distinct from those capable of pore-formation, including fragments of clostridial neurotoxins, such as either botulinum or tetanus toxins, when introduced into the cytoplasm of target cells are capable of inhibiting the transport of the IMPs in RMVs to the surface of the cell, so reducing the concentration of those IMPs at the cell surface. In particular it has been found that fragments of tetanus toxin and botulinum types A, B, C_1, D, E, F and G are particularly suitable. An example of such a molecule is that portion of a clostridial neurotoxin known as the H_2L fragment, in which the neuronal targeting activity of the carboxyterminal half of the heavy chain of the toxin has been removed, leaving the amino terminal half disulphide - linked to the light chain. Another example would be the Light chain of a clostridial neurotoxin such as the Light chain of the botulinum neurotoxin type B, in particular those portions of the molecule which have Zn^{++} dependent metalloprotease activity.

The invention therefore includes an agent of the following structure;

Domain B--Domain T--Domain E

The Domains are covalently linked by linkages which may include

appropriate spacer regions between the Domains.

In one embodiment of the invention Domain B is a binding
molecule capable of binding to a Binding Site on the target cell
which undergoes endocytosis, Domain T is the Heavy chain of a
botulinum neurotoxin or fragments thereof and Domain E is the
Light chain of a botulinum neurotoxin or fragments thereof.
Domains T and E can be from the same or different serotypes of
C.botulinum.

In another embodiment of the invention Domain B is a binding
molecule capable of binding to a Binding Site on the target cell
which undergoes endocytosis, Domain T is the Heavy chain of a
tetanus neurotoxin or fragments thereof and Domain E is the
Light chain of a botulinum neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding
molecule capable of binding to a Binding Site on the target cell
which undergoes endocytosis, Domain T is the Heavy chain of a
botulinum neurotoxin or fragments thereof and Domain E is the
Light chain of a tetanus neurotoxin or fragments thereof.

In another embodiment of the invention Domain B is a binding
molecule capable of binding to a Binding Site on the target cell
which undergoes endocytosis, Domain T is the Heavy chain of a
tetanus neurotoxin or fragments thereof and Domain E is the
Light chain of a tetanus neurotoxin or fragments thereof.

It is to be understood that this invention includes any
combination of toxin molecules or fragments of toxin molecules
from the same or different organisms which have the functions
described.

When the agent is administered to an organism the concentration
of IMPs at the surface of the target cell is reduced. This can
lead to a number of desired effects including reduced intake of
a metabolite or ion into or across the cell, reduction in
response of the target cell to a signalling molecule, or change
in the immune response of the organism.

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EXAMPLES

EXAMPLE 1

3T3-LI fibroblasts are trypsinized into suspension and are electroporated at 300V/ cm, 960 mF with a time constant of 11 - 11.5 msecs, using a Bio-Rad Gene Pulser with capacitance extender, in the presence or absence of 1mM botulinum neurotoxin-B (BoNT-B). Following electroporation the cells are allowed to adhere and are maintained in monolayer culture at 37°C in 24-well plates for 72 h. The cells are then washed and incubated for 5 min at 37°C in the presence or absence of 5nM insulin-like growth factor type 1 (IGF-1), followed by standing on ice for 5 min. The supernatant is aspirated from the cells and replaced with ice-cold 1.5 nM ^{125}I-transferrin (sp. act. 47 Tbq / mmol). Non-specific binding is estimated in parallel incubations performed in the presence of a 100 fold molar excess of non-radioactive transferrin. After 2h the supernatant is removed, and following 3 washes with ice-cold buffer the cell layer is digested in 1N NaOH, and the bound ^{125}I-transferrin measured using a LKB1275 minigamma gamma counter. Up-regulation of transferrin-binding is calculated as the specific ^{125}I-transferrin binding in the presence of IGF -1 expressed as a percentage of the specific binding in the absence of IGF-1.

Table 1 shows that there is a reduced elevation of ^{125}I-transferrin binding in response to IGF-1 in BoNT-B treated cells compared to control. This indicates that introduction of BoNT-B into the cytosol of 3T3-LI fibroblasts inhibits the IGF-1 stimulated up-regulation of transferrin receptors in these cells.
Triton-X-114-soluble proteins extracted from the 3T3-LI fibroblasts digests are analysed by Western blotting using a polyclonal antibody raised against a peptide sequence SEQ.ID.1: (QQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWK NLK) identified in a secretory vesicle protein of neurosecretory cells. This anti-vesicle antibody shows reduced reactivity with the relevant doublet band in samples from BoNT-B-treated fibroblasts, which have no reported neurosecretory activity.

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Thus, BoNT-B is modifying vesicle (presumably RMV) structure in
3T3-LI fibroblasts concurrently with inhibiting up-regulation
of transferrin receptors.

EXAMPLE 2

3T3-LI fibroblasts are electroporated in the presence or absence
of 0.5 mM botulinum neurotoxin-A (BoNT-A) using conditions
identical to those given in example 1. IGF-1 stimulation of
^{125}I-transferrin binding is assayed in treated and untreated
cells as described in example 1.

The results in table 2 show that BoNT-A treatment of 3T3-LI
fibroblasts abolishes the up regulation of ^{125}I-transferrin
binding seen in response to IGF-1.   This indicates that
introduction of BoNT-A into the cytosol of 3T3-LI fibroblasts
inhibits the IGF-1 stimulated up-regulation of transferrin
receptors in these cells.

EXAMPLE 3

3T3-LI fibroblasts are electroporated in the presence or absence
of 0.5mM of the H_2L-fragment of BoNT-A (H_2L-A) using conditions
identical to those given in example 1.   This fragment is
produced from the neurotoxin, serotype A, of C botulinum by
limited proteolysis using tosylphenylalaninechloromethane-
treated trypsin.   The H_2L complex is then purified by
chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; &
Melling, J; 1985, Eur J Biochem 151, 17-82). Electroporation
is performed as described in example 1 as is the measurement
IGF-1 stimulation of ^{125}I-transferrin binding in treated and
untreated cells.

The results in table 3 show that H_2L-A treatment of 3T3-LI
fibroblasts inhibits the up-regulation of ^{125}I-transferrin
binding seen in response to IGF-1.   This indicates that
introduction of the H_2L-A fragment of botulinum neurotoxin-A
into the cytosol of 3T3-LI fibroblast inhibits the IGF-1
stimulated up-regulation of transferrin receptors in these

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cells.

EXAMPLE 4

3T3-LI adipocytes are differentiated from 3T3-LI fibroblasts by
treatment with dexamethasone, 3-isobutyl-1-methylxanthine and
insulin as described (Frost, SC & Lane MD, 1985, J Biol Chem
260, 2646 -2652). The 3T3-LI adipocytes 7 days after
differentiation are treated with Botulinum neurotoxin serotype
A diluted into Dulbecco's modified Eagles medium containing
serum and filter sterilised (final concentration BoNT A:200nM).
Toxin treated and control cells are incubated at 37°C for 45
hours in 8% CO_2. The cells are then washed twice and incubated
in 8% CO_2 for 2 hours in serum-free Dulbecco's modified Eagle's
medium after which the cells are washed in Krebs Ringer
phosphate and incubated in either Krebs Ringer phosphate (basal
uptake) or Krebs Ringer phosphate containing 100nM insulin
(stimulated uptake) for 15 minutes at 37°C. Glucose uptake is
initiated by the addition of [³H] 2-deoxyglucose (14.2KBq, 10uM
glucose). After 10 minutes at 37°C the reaction is stopped by
aspiration of the glucose solution and rapid washing with ice
cold phosphate buffered saline. Cells are lysed by the addition
of 0.2N NaOH and the solution neutralised by the addition of
0.2N HCl. Uptake of [³H] 2-deoxyglucose is measured by liquid
scintillation counting in optiphase scintillant using a Wallac
1410 liquid scintillation counter.

It is known that clostridial neurotoxins are able to enter
certain neurosecretory cells (for example PC12 cells) via a low
affinity receptor if high concentrations of the neurotoxin are
incubated with the cells for prolonged periods. This process
appears to use a pathway via a receptor which is distinct from
the highly specific and high affinity receptor present at the
neuromuscular junction. Additionally it has been reported that
certain clostridial toxins have effects on phagocytic cells,
such as macrophages, where entry into the cell is presumed to
be via the specific phagocytic activity of these cells.
Generally, it is recognised, however, that the neuronal
selectivity of clostridial neurotoxins is a result of a very
selective binding and cell entry mechanism. It is, therefore,

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the surprising finding of these studies, that incubation of 3T3-
LI adipocytes with botulinum neurotoxin-A, as described, causes
a marked inhibition of insulin-stimulated up-regulation of [³H]
2-deoxyglucose transport (table 4). It is known that insulin-up
regulation of glucose transport in adipocytes is a result of
movement of glucose transporter proteins from intracellular
pools (RMVs) to the cell surface. Thus, this result
demonstrates that botulinum neurotoxin-A inhibits the insulin-
stimulated movement of glucose transporters in RMVs to the cell
surface of adipocytes.

EXAMPLE 5

3T3-LI adipocytes are trypsinised and a suspension of the cells
is electroporated in the presence or absence of Botulinum B
(0.32mM). A 960 mF capacitor is used for electroporation
producing a pulse strength of 300 V/cm; the time constant is 11-
12 ms. After electroporation the cells are washed and plated
out in a 6 well plate with media and serum. The cells are
incubated at 37°C in a humidified atmosphere (air/CO_2;
92.5%/7.5%) for 72 h. At the end of this period, the cells are
washed and extracted into 0.1N NaOH. Following neutralisation
of the extract with 0.1N HCl the membrane proteins are
partitioned into Triton X-114 and subsequently analysed by
Western blotting using the anti-vesicle antibody described in
example 1. The surprising finding of this study is that
electroporation of botulinum neurotoxin into the cytosol of
adipocytes results in a modification of vesicle (presumably RMV)
structure as evidenced by reduced reactivity of the antibody
with the relevant doublet band on samples from botulinum
neurotoxin-B treated cells.

EXAMPLE 6

In this example, an agent is synthesized to regulate the cell
surface expression of the insulin-dependent glucose transporter
of adipocytes.

The binding Domain (B) for the agent in this example is insulin-

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like growth factor II, which is purified from the conditioned
medium of BRL-3A cells as described (Marquette, H; Todaro, GJ;
Henderson, LE & Oroszlan, S, 1981, J Biol. Chem $\underline{256}$ 2122-2125).

The translocating Domain (T) is prepared from the neurotoxin,
serotype A, of *C. botulinum* by limited proteolysis of the
neurotoxin with tosylphenylalaninechloromethane-treated trypsin.
The fraction containing Domain T is then purified by
chromatography on Sephadex G-200 (Shone, CC; Hambleton, P; &
Melling, J; 1985, Eur. J. Biochem. $\underline{151}$, 75-82). This fraction
is then applied in phosphate/borate buffer, pH 8.4 onto a
quaternary aminoethyl-Sephadex column, and incubated on the
column at 4°C overnight with 2M urea and 0.1M dithiothreitol.
The column is then washed with buffer containing 2M urea and 10mM
dithiothreitol. Domain T is then eluted using phosphate/borate
buffer containing 2M urea and 10mM dithiothreitol and a stepwise
gradient of NaCl from 0.1 to 0.2M (Poulain, B; Wadsworth, JDF;
Maisey, EA; Shone, CC; Melling, J; Tauc, L; & Dolly, JO, 1989,
Eur. J. Biochem. $\underline{185}$, 197-203). The clostridial neurotoxins
are disulphide-linked dichain proteins consisting of a heavy
chain and a light chain (Simpson, LL, 1986, Ann. Rev.
Pharmicol. Toxicol. $\underline{26}$, 427-453). It should be noted that the
Domain T, produced in the manner given, is equivalent to a
fraction of the heavy chain of the neurotoxin referred to as H_2.

The effector Domain (E) is prepared from the neurotoxin of *C.
tetani* by isoelectric focusing in a sucrose gradient with
ampholyte under reducing conditions in 2M urea (Weller, U;
Dauzenroth, M-E; Meyer zu Heringdorf, D & Habermann, E, 1989,
Eur. J. Biochem., $\underline{182}$, 649-656). It should be noted that the
Domain E produced in the manner given is equivalent to the light
chain of the neurotoxin, commonly referred to as LC.

Domains E and T are mixed together in equimolar proportions
under reducing conditions and covalently coupled by repeated
dialysis, at 4°C with agitation, into physiological salt
solution in the absence of reducing reagents. Any remaining
free sulphydryls are derivatized by the addition of 150mM
iodoacetamide for 30 min at 4°C in the dark. The conjugated E-T

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product is purified by size exclusion chromatography on Sephadex
G-150 using potassium phosphate buffer, pH 7.0. Finally, Domain
B is coupled to the E-T complex using N-succinimidyl 3-(2-
pyridylothio) proprionate (SPDP). The E-T complex (5 mg) is
dissolved in 1 ml of phosphate buffered saline (PBS), and to
this is added   200 mg of SPDP dissolved in 0.5 ml of absolute
ethanol. After reacting the mixture at room temperature for 30
mins, the 2-pyridyldisulphide-substituted peptide is separated
from excess SPDP by gel filtration through Sephadex G25. Domain
B is similarly treated, but using less SPDP (20 mg in 0.2 ml
ethanol). The substituted Domain B is again harvested from a
Sephadex G25 column, and is then reduced by the addition of
dithiothreitol to a final concentration of 0.05M. Excess
reducing agent is removed by gel filtration on Sephadex G25.
Equal portions (w/w) of the substituted E-T complex and the
substituted and reduced Domain B are then mixed together and
left at 4°C for 18h. The agent is then purified by
chromatography on Sephadex G-150 using potassium phosphate
buffer, pH 7.0.

The agent, prepared as described, is then tested for its ability
to inhibit the insulin-stimulated increase in glucose
transporter expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes
are differentiated from 3T3-L1 fibroblasts by treatment with
dexamethasone, 3-isobutyl-1-methylxanthine and insulin as
described (Frost, SC & Lane MD, 1985, J Biol Chem 260, 2646-
2652), and are used between 8 and 12 days after initiation of
differentiation. Cells are incubated with or without the agent
for 90 min at 37°C. Cells are then incubated for 2 hours in
serum-free Dulbecco's modified Eagle's medium at the beginning
of each experiment. Insulin-treated cells are then exposed for
10 minutes to 10^{-7}M insulin which is added from a stock 1.6 x 10^{-4}M solution. After treatment as described above the cells are
washed quickly with Krebs-Ringer phosphate at 37°C and the
uptake of [3H] 2-deoxyglucose (14.2 KBq;10 mM) in Krebs Ringer
phosphate at 37°C with or without 10^{-7}M insulin over a 10 minute
period is then measured. The reaction is stopped by aspiration
of the glucose solution and rapid washing with ice cold
phosphate buffered saline. Cells are lysed by the addition of

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0.2M sodium hydroxide and the solution is neutralised by the
addition of 0.2M hydrocholoric acid prior to scintillation
counting in Optiphase scintillant using a Wallac 1410 liquid
scintillation counter.

EXAMPLE 7

In another example of the invention Domains E and T are produced
from the same serotype of botulinum neurotoxin and are produced
already coupled together. The neurotoxin, serotype A, of C.
botulinum is subjected to limited proteolysis using
tosylphenylalaninechloromethane-treated trypsin. The E-T
complex is then purified by chromatography on Sephadex G-200
(Shone, CC; Hambleton, P; & Melling, J 1985, Eur. J. Biochem.
151, 75-82).

It should be noted that this fragment is equivalent to that
referred to as the H_2L fragment. Any remaining free sulphydryls
are derivatized by the addition of 150mM iodoacetamide as
described in example 6. The binding Domain (B) is insulin-like
growth factor II prepared as described in Example 6, and coupled
to the E-T complex using SPDP as described. The activity of the
agent on the expression of insulin-dependent glucose transport
in adipocytes is tested as described in Example 6.

EXAMPLE 8

In another example of the invention, an agent for the regulation
of the cell surface expression of the CR1 receptor for
complement fragment C3b in neutrophils (CD 35) is synthesized
in the following manner. The B Domain is prepared from the
SHCL3 monoclonal antibody to the leukocyte adhesion molecule
P150,95. The E and T Domains are prepared from botulinum
neurotoxin, serotype A, ready coupled, as described in Example
7, and are coupled to Domain B, as described in that example.

The preferred method for testing the activity of the agent on
neutrophil cell surface expression of CR1 (CD35) is using the
whole blood lysing technique. EDTA anticoagulated whole blood

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from normal donors is treated with the agent for 4 hours and
then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-Phe
diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control
cells are incubated with PBS. The blood is then incubated for
30 minutes at room temperature with 10ml of Phycoerythrin
conjugated monoclonal antibody antiCD35 (Serotec:MCA 554PE), red
blood cells are lysed using Becton Dickinson lysing fluid,
leukocytes washed with PBS and resuspended in 2% formaldehyde
in PBS. Suface bound PE is analysed by flow cytometry using a
FACScan (Becton Dickinson) equipped with Lysys II software.

EXAMPLE 9

In another example of the invention, an agent for the regulation
of the cell surface expression of the leukocyte adhesion
molecule Mac-1 (CD IIb) is synthesized in the following manner.
The B Domain is prepared from the SHCL3 monoclonal antibody to
the leukocyte adhesion molecule P150,95 by standard
methodologies using pepsin, and is purified by gel filtration
(Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981,
Biochemistry 20, 4229-4238). The E and T Domains are prepared
from botulinum neurotoxin, serotype A, ready coupled, as
described in Example 7, and are coupled to Domain B as described
in that example.

The preferred method for testing the activity of the agent on
neutrophil cell surface expression of Mac-1 (CD11b) is using the
whole blood lysing technique. EDTA anticoagulated whole blood
from normal donors is treated with the agent for 4 hours at 37°C
and then activated for 30 minutes at 37°C using 10^{-6}M fMet-Leu-
Phe diluted in PBS from a stock of 10^{-2}M made up in DMSO. Control
cells are incubated with PBS. The blood is then incubated for
30 minutes at room temperature with 10ml of fluoroscein
isothiocyanate conjugated monoclonal antibody antiCD11b
(Serotec:MCA 551F). The red blood cells are lysed using Becton
Dickinson lysing fluid, the leukocytes washed with PBS and
resuspended in 2% formaldehyde in PBS. Surface bound FITC is
analysed by flow cytometry using a FACScan (Becton Dickinson)
equipped with Lysys II software.

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EXAMPLE 10

In another example of the invention, an agent for the regulation of the cell surface content of Na$^+$ channels in the apical membrane of bladder epithelium is synthesized in the following manner. The B Domain is prepared from a high affinity monoclonal antibody to a cell surface marker of bladder epithelial cells by standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL; and Papahadjopoulos, D, 1981, Biochemistry 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 7, and are coupled to Domain B as described in that example.

The effect of the agent on aldosterone-stimulated increases in amiloride-sensitive Na$^+$- channels is tested using urinary epithelial cells. Bladder epithelial cells, prepared as primary cultures from rat bladder (Johnson, MD; Bryan, GT; Reznikoff, CA;1985, J Urol 133, 1076-1081), are incubated with or without the agent for 90 mins at 37°C. Aldosterone-treated cells are then exposed for 1h to aldosterone. After treatment as described, the cells are rapidly washed and the amiloride-sensitive uptake of ^{22}Na$^+$ over a 5 min incubation at 37°C is measured.

EXAMPLE 11

In another example of the invention, an agent for regulating antigen-presentation by B-cells is synthesized in the following manner. The B Domain is prepared from the pan B cell monoclonal antibody LL2 using standard methodology using pepsin, and is purified by gel filtration (Martin, FJ; Hubbell, WL & Papahadjopoulos, D, 1981, Biochemistry, 20, 4229-4238). The E and T Domains are prepared from botulinum neurotoxin, serotype A, ready coupled, as described in Example 3, and are coupled to Domain B also as described in that example.

The effect of the agent on antigen-presentation is tested using the murine B lymphoma cell-line TA3. These cells are first

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incubated with the agent for 90 mins at 37°C, and then hen egg lysozyme (HEL) is added and the incubation continued for 2h at 37°C. The TA3 cells are then fixed and washed before culture with the I-A^K- restricted HEL46-61 specific T-cell hybridoma 3A9 (Lorenz, RG & Allen, PM, 1989, Nature 337, 560). The supernatant from the 3A9 cells is tested for its ability to support growth of the IL-2-dependent cell line, CTLL. Proliferative responses are measured by the incorporation of ^{3}H-thymidine over a 3h period following 2 days of culture with the supernatant.

The examples described above are purely illustrative of the invention. It should be clear to those skilled in the art that any combination of the three domains are within the scope of this invention. In synthesising the agent the coupling of the T-E component of the invention to the targeting component is achieved via chemical coupling using reagents and techniques known to those skilled in the art. Thus, although the examples given use exclusively the SPDP coupling reagent any other coupling reagent capable of covalently attaching the targeting component of the reagent and known to those skilled in the art is covered in the scope of this application. Similarly it is evident to those skilled in the art that either the DNA coding for either the entire agent or fragments of the agent could be readily constructed and, when expressed in an appropriate organism, could be used to produce the agent or fragments of the agent. Such genetic constructs of the agent of the invention obtained by techniques known to those skilled in the art are also covered in the scope of this invention.

EXPLOITATION IN INDUSTRY

The agent described in this invention can be used *in vivo* either directly or as a pharmaceutically acceptable salt or ester in a method of treatment for a variety of pathophysiological states.

For example, one form of the agent can be used in a method of treatment for glucose metabolism disorders by limiting the

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uptake of glucose by certain cells. A specific example of this would be the use of a form of the agent in a method of treatment for clinical obesity by limiting the uptake of glucose by adipose cells and hence reducing accumulation of lipid in these cells.

In another example a form of the agent can be used in a method of treatment for hypertension by regulating the ion uptake by kidney cells and hence controlling the output of fluid from these organs.

In yet another example a form of the agent can be used in a method of treatment for inflammation by controlling the response of target cells to external signals which trigger the inflammatory response.

In yet another example a form of the agent can be used in a method of treatment for immune disorders by controlling the presentation of peptide sequences by antigen presenting cells to the effector cells of the immune system.

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TABLE 1

IGF-1 Up-Regulation Of ^{125}I-Transferrin Binding In 3T3-LI Fibroblasts

Treatment	IGF-1	% basal binding ± SD*
Control	-	100 ± 28 (n=3)
	+	258 ± 46 (n=3)
BoNT-B	-	100 ± 8 (n=3)
	+	149 ± 27 (n=3)

* Specific binding of ^{125}I-transferrin to 3T3-LI fibroblasts expressed as a percentage of the specific binding in the absence of IGF-1.

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TABLE 2

IGF-1 Up-regulation of ^{125}I-transferrin binding in 3T3-LI
fibroblasts

Treatment	IGF-1	% basal binding $\pm$ SD*
Control	-	100 $\pm$ 28 (n=3)
	+	258 $\pm$ 46 (n=3)
BoNT-A	-	100 $\pm$ 44 (n=3)
	+	149 $\pm$ 10 (n=3)

*   Specific binding of ^{125}I-transferrin to 3T3-LI
fibroblasts expressed as a percentage of the specific binding
in the absence of IGF-1.

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TABLE 3

IGF-1 Up regulation of ^{125}I-transferrin binding in 3T3-LI
fibroblasts

Treatment	IGF-1	% basal binding $\pm$ SD*
Control	-	100 $\pm$ 28 (n=3)
	+	258 $\pm$ 46 (n=3)
H$_2$L-A	-	100 $\pm$ 15 (n=3)
	+	134 $\pm$ 60 (n=3)

*   Specific binding of ^{125}I-transferrin to 3T3-LI
fibroblasts expressed as a percentage of the specific binding
in the absence of IGF-1.

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TABLE 4

Uptake of $[^3H]$ -2-deoxyglucose by 3T3-LI adipocytes

	Basal	Insulin-stimulated
Control	1655 ± 67 (n=3)	$14\ 328 \pm 264$ (n=3)
BoNT-A treated	2306 ± 49 (n=3)	5587 ± 322 (n=3)

The results are the means $\pm$ SEM of triplicate determinations and
are given as the total dpm taken up by the cell monolayer during
a 10 min incubation.

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SEQUENCE LISTING

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        (D) STATE: WILTSHIRE
        (E) COUNTRY: UNITED KINGDOM
        (F) POSTAL CODE (ZIP): SP5 3BN

    (ii) TITLE OF INVENTION: NOVEL AGENT FOR CONTROLLING CELL ACTIVITY

    (iii) NUMBER OF SEQUENCES: 1

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 9305735.4
        (B) FILING DATE: 19-MAR-1993

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(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 62 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
1   5   10   15

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
            20   25   30

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
        35   40   45

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
        50   55   60

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CLAIMS

1.   An agent for controlling the interaction of a cell
        with its external environment by controlling the
        transport of Integral Membrane Proteins to the
        membrane of the cell in Recyclable Membrane
        Vesicles.

2.   An agent according to Claim 1 for the control of the
        level of the IMP responsible for the transport of a
        metabolite across the cell membrane, so controlling
        the availability of that metabolite within the cell.

3.   An agent according to Claim 1 for the control of the
        level of the IMP responsible for the transport of a
        metabolite across the cell membrane into the cell
        and out of the cell, so controlling the transport of
        that metabolite through the cell.

4.   An agent according to Claim 1 for the control of the
        level of the IMP responsible for the selective
        permeability of the plasma membrane of the cell to
        an ion, so modulating the concentration of that ion
        within the cell.

5.   An agent according to Claim 1 for the control of the
        level of the IMP responsible for the recognition of
        a signalling molecule, so modulating the
        responsiveness of the cell to that signalling
        molecule.

6.   An agent according to Claim 1 for the control of the
        level of the IMP responsible for the transduction of
        signals across the cell membrane following binding
        to the membrane of a signalling molecule, so
        modulating the responsiveness of the cell to that
        signalling molecule.

7.   An agent according to Claim 1 for the control of the

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level of the IMP responsible for the display on the cell surface of peptide fragments derived from ingested antigens.

8.   An agent according to any preceding claim which has target specificity for target cell types so that the scope of the effect of the agent is limited to said cell types.

9.   An agent according to any preceeding claim which comprises three Domains B,T and E linked together in the following manner:

                     Domain B--Domain T--Domain E

Where

Domain B is the Binding Domain which binds the agent to a Binding Site on the cell which undergoes endocytosis to produce an endosome

Domain T is the Translocation Domain which translocates the agent (with or without the Binding Site) from within the endosome across the endosomal membrane into the cytosol of the cell

Domain E is the Effector Domain which inhibits the ability of the RMVs to transport the IMPs to the surface of the cell.

10.   An agent according to Claim 9 which comprises three Domains B,T and E linked together in the following manner;

                 Domain B--X--Domain T--X--Domain E

Where X is either a spacer molecule or a covalent linkage but at least one X is a spacer molecule.

11.   An agent according to Claims 9 or 10 in which Domain B, the Binding Domain, binds to a Binding Site which is characteristic of a particular cell type.

12.   The agent according to any preceding claim in which

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Domain B comprises a monoclonal antibody to a
surface antigen on the target cell capable of
undergoing endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a
toxin molecule which translocates the agent from the
resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain
fragment of a toxin molecule functionally distinct
from Domain T with Zn^{++} dependent proteolytic
activity.

13.   The agent according to any preceding claim in which

Domain B comprises a ligand to a cell surface
receptor on the target cell capable of undergoing
endocytosis to produce an endosome
Domain T comprises a domain or domain fragment of a
toxin molecule which translocates the agent from the
resulting endosome into the cytosol of the cell
Domain E comprises a different domain or domain
fragment of a toxin molecule functionally distinct
from Domain T with Zn^{++} dependent proteolytic
activity.

14.   An agent according to claim 13 which affects the rate
of glucose uptake by adipose cells in response to
insulin in which

Domain B is a ligand to the insulin-like growth
factor II receptor
Domain T is the domain or domain fragment of the
botulinum neurotoxin Heavy chain responsible for
translocation of the toxin across the cell membrane
Domain E is the domain or domain fragment of the
Light chain of botulinum neurotoxin having Zn^{++}
dependent metalloprotease activity.

15.   An agent according to any of claims 9 to 14 in which
Domains T and E are obtained from Clostridial

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neurotoxin.

16.   A process for the manufacture of an agent according
        to any preceding claim which comprises the covalent
        attachment of three Domains B,T and E in the
        following manner;

                Domain B--Domain T--Domain E

        Where
        Domain B is the Binding Domain which binds the agent
        to a Binding Site on the cell which undergoes
        endocytosis to produce an endosome
        Domain T is the Translocation Domain which
        translocates the agent (with or without the Binding
        Site) from within the endosome across the endosomal
        membrane into the cytosol of the cell
        Domain E is the Effector Domain which inhibits the
        ability of the RMVs to transport the IMPs to the
        surface of the cell.

17.   A process for the manufacture of an agent according
        to Claim 16 which comprises the covalent attachment
        of three Domains B,T and E in the following manner;

                Domain B--X--Domain T--X--Domain E

        Where X is either a spacer molecule or a covalent
        linkage but at least one X is a spacer molecule.

18.   A process according to Claims 16 or 17 in which
        Domain B, the Binding Domain, binds to a Binding
        Site which is characteristic of a particular cell
        type.

19.   A method of making the agent according to any
        preceding claim comprising constructing a genetic
        construct which codes for the agent, incorporating
        said construct into a host organism and expressing

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the construct to produce the agent.

20.   Use of the agent or a pharmaceutically acceptable
        salt thereof according to any preceding claim for
        the treatment of glucose metabolism disorders.

21.   Use of the agent or a pharmaceutically acceptable
        salt thereof according to any preceding claim for
        the treatment of clinical obesity.

22.   Use of the agent or a pharmaceutically acceptable
        salt thereof according to any preceding claim for
        the treatment of hypertension.

23.   Use of the agent or a pharmaceutically acceptable
        salt thereof according to any preceding claim for
        the treatment of inflammatory disorders.

24.   Use of the agent or a pharmaceutically acceptable
        salt thereof according to any preceding claim for
        the treatment of immune system disorders.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   A61K47/48   C07K15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 00099 (FOSKNINGSSTIFTELSEN DET NORSKE RADIUMHOSPITAL) 9 January 1992 see page 3, line 2 - line 14; claim 1 ---	1-24
A	WO,A,93 04191 (NEORX CORPORATION) 4 March 1993 see page 25, paragraph 2; claims 11,2,4,8 ---	1-24
A	WO,A,91 17173 (CYTOGEN CORPORATION) 14 November 1991 see claim 1 ---	1-24
	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 1994	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Berte, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	BIOCHEMISTRY, vol.33, 1994, EASTON, PA US pages 2604 - 2609 SIMS K. KOCHI ET AL. 'THE EFFECTS OF PH ON THE INTERACTION OF ANTHRAX TOXIN LETHAL AND EDAMA FACTORS WITH PHOSPHOLIPID VESICLES' See abstract -----	9-24

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

   please see enclosure!

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9200099	09-01-92	AU-A-	8000191	23-01-92
		CA-A-	2086342	28-12-91
		EP-A-	0542756	26-05-93
		JP-T-	6503552	21-04-94
WO-A-9304191	04-03-93	NONE		
WO-A-9117173	14-11-91	US-A-	5196510	23-03-93
		EP-A-	0527954	24-02-93